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We have demonstrated that certain transduction peptides such as 12 lysines and 12 arginines can facilitate internalization into breast tumor lines with higher efficiency than smaller polymers of cationic amino acids. In addition, we have demonstrated that PTD-Sma34 worked in conjunction with Ad.TRAIL gene transfer to induce breast tumor apoptosis. We also have demonstrated that membrane bound TRAIL worked more effectively than soluble (secreted) TRAIL to induce breast cancer apoptosis. Moreover, we have demonstrated that co-administration of Ad.TRAIL with PTD-Smac34 resulted in not only enhanced adenoviral transduction of the tumors, but resulted in a stronger apoptotic effect. Finally, we have initiated studies to identify breast cancer specific tumor lines by screening a peptide phage display library both in cell culture as well as in nude mice bearing xenografts. Initial results in prostate tumors has demonstrated the feasibility of this approach and suggests breast tumor specific internalization peptides can be identified.

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Introduction

In the United States, breast cancer is the leading cause of death for women between the ages of 45 and 55. As the most common malignancy facing women, during 2001 it was estimated that 192,000 women were diagnosed with breast cancer and 40,200 died from the disease. Current management approaches for breast cancer vary based on stage, however therapeutic approaches can include various combinations of surgery, radiation, chemotherapy and hormone therapy. Despite these approaches, the significant mortality from breast cancer has called attention to additional therapies which target dysregulated pathways present in the tumor cells. Alterations in estrogen receptor-mediated signaling, mutations in p53, C erb 2 overexpression, upregulated p-glycoprotein (gp170) and dysfunction of apoptotic pathways have been associated with poor prognosis in breast cancer. Gene therapy approaches have attempted to target these anomalies present in malignant tissue, however, the efficiency of gene transfer has proven to be the rate-limiting step. Thus there is a need for more efficient systems for intra-cellular delivery of apoptotic or tumor suppressor proteins. One approach for the efficient intra-cellular delivery of proteins involves the use of protein transduction. Amino acid sequences within HIV Tat and *Drosophila* Antennapedia (Antp) proteins, termed PTDs for protein transduction domains, have been shown to facilitate efficient receptor and energy independent internalization of large protein complexes into a wide variety of cell types in culture and *in vivo*. Recently, we have identified a class of cationic peptides similar to HIV TAT and Antp PTD, rich in arginine and lysine, able to facilitate internalization of marker protein complexes into a wide variety of cell types including breast tumor cell lines. In preliminary experiments, we have demonstrated that certain transduction peptides can facilitate internalization into breast tumor lines 5-fold more efficiently than the HIV Tat PTD. We also have previously demonstrated the feasibility of using PTDs for the treatment of cancer in murine tumor models, where a specific cationic peptide was able to efficiently transduce and kill tumor cells following intra-tumoral injection. Intra-tumoral injection of a mitochondrial disruption peptide, KLAK, fused to a cationic transduction domain resulted in significant murine tumor apoptosis and complete tumor regression. *In vitro*, a peptide derived from the C-terminal negative regulatory domain of p53 which leads to stabilization of wild-type and certain mutants of p53, appears to potentiate the apoptotic effects of etoposide or TRAIL in the MCF-7 and ZR75-1 breast cancer lines following PTD-mediate internalization. Similar effects have been observed in these breast cancer lines when using PTD-mediated delivery of NF-kB inhibitory peptides or peptides derived from the amino terminus of Smac, a protein able to block the anti-apoptotic effect of IAPs (Inhibitors of Apoptosis). The activity of these peptide cargoes *in vitro* against breast cancer cells, coupled with the ability of PTDs to deliver their cargoes with high efficiency to cells *in vivo* holds the potential for generating novel therapies that bypass the limitations of conventional approaches. In addition to the cationic, non-specific transduction peptides, we also have developed a method for screening for tissue-targeted transduction peptides using an M13 peptide phage display library. Using this method we have identified peptides able to transduce human synovial fibroblasts and airway epithelial cells, as well as one peptide able to target prostate tumor lines specifically. Thus the overall goal of the proposal is to optimize and utilize peptide transduction for efficient delivery of therapeutic peptides and eventually proteins into breast cancer cells. This approach could be used for treatment of not only localized tumor by direct injection into the breast, but also could be used for treatment of metastatic disease. The successful completion of the proposed studies should lead to identification of the optimal transduction peptides for internalization of a variety of therapeutic agents, including peptides, proteins and drugs into breast cancer cells that could be used clinically.

Research Progress

Objective 1: To identify the optimal cationic transduction domain for breast cancer cells as well as identify breast specific transduction domains.

Task 1. A panel of peptides, 4 to 12 amino acids in length and enriched for arginines and lysines will be screened for their ability to transduce two different breast lines, MCF-7 and ZR75-1. The screening will be performed using biotinylated peptides coupled to two different marker complexes, avidin-ß-Gal and avidin-488. (Months 1-6, Year 1)

Progress: We have screened a panel of arginine and lysine rich transduction peptides for transduction of MCF-7 and ZR75-1 cells in culture by FACS. For the experiment shown below, biotinylated peptides were coupled to Avidin-488 and the transduced MCF-7 and ZR75-1 cells analyzed by flow cytometry. The fold increase in fluorescence is shown. Consistent with other tumor models, the most effective peptide for transduction was a peptide containing lysines followed closely by peptides containing arginines. However, unlike other types of tumors, the efficiency of transuduction was less for short polycationic peptides (e.g. 6R and 8K), requiring longer polymers of positively charged amino acids such as 12K and 12R. These results are similar to that observed for T cells where 12 R and 12K are more effective than 8R and 8K. In contrast, analysis of transduction of prostate, head and neck tumors, and gliomas has shown that 6R and 8K are optimal Thus 12K or 12R will be used for all subsequent experiments.

Fold Increase in Transduction

	MCF-7	ZR75-1
PTD-5	1.91	2.77
TAT	7.56	10.53
4R	2.08	1.87
6R	4.56	8.36
8R	25.29	18.86
10R	32.11	19.88
12R	33.11	25.26
4K	1.22	1.35
6K	2.36	4.62
8K	13.02	6.23
10K	13.02	17.62
12K	34.53	16.30
5RQ	1.65	1.70
8RQ	3.93	4.20
11RQ	27.51	13.13

Task 2. The four best peptides for transduction of breast cancer cells will be characterized for intracellular localization by confocal microscopy following internalization by conjugation to avidin-488. The HIV Tat and PTD-5 peptides will be used as positive controls. (Months 3-9, Year 1)

Progress: The 8K and 6R peptide are found to present in both the cytoplasm and nucleus of the cell. However, since the 8K peptide resembles a nuclear localization signal, it is found in the nucleus at a higher percent than 6R. Given our recent PTD screening results (see above) we are now currently examining the intracellular localization of the 12K and 12R peptides.

Task 3. The optimal peptides (maximum of 2), based on their ability to deliver the ß-gal and 488 marker complex to the cytoplasmic and/or nucleus of the different breast cancer lines in culture will be examined for ability to transduce breast cancer cells *in vivo*. Nude mice will be inoculated subcutaneously with MCF-7 and ZR75-1 cells. When the tumors reach palpable size, the peptide-avidin-ß-gal complexes will be injected intra-tumorally and the extent of transduction examined three hours post-injection by X-gal staining of tumor sections. The HIV and PTD-5 peptides will be used as a positive control. 3 mice will be used per treatment group. However, different doses of peptide-marker conjugates may have to be tested. (Months 6-12, Year 1)

Progress: In initial experiments, the 8K peptide was more effective than other peptides for transduction of ZR75-1 tumors in nude mice. Given the recent results demonstrating the more effective transduction of breast tumor lines with larger polymers of lysine and arginine, these experiments are currently being repeated.

Task 4. An M13 peptide phage display library will be used for screening for novel transduction peptides able to facilitate internalization into breast cancer lines in culture. The screen will be performed on MCF-7 and ZR75-1 cells with three rounds of screening. The phage that are isolated with be panned against HeLa cells to eliminate any non-specific internalizing peptides. (Months 1-12, Year 1).

Progress: This screen is currently in progress. We have completed two rounds of screening with evidence of enrichment. A similar screen being performed on prostate tumors has shown a significant increase in tumor specific, intracellular M13 phage following IV injection. This result in prostate tumor suggests that similar enrichment and positive results can be obtained following completion of four rounds of screening.

Task 5. The ability of the breast screened transduction peptides to facilitate internalization of avidin-ß-gal and avidin-488 into breast cancer lines will be examined. In addition, the specificity of transduction will be evaluated by examining transduction of HeLa, Saos-2 and LNCaP cells. (Months 1-6, Year 2)

Progress: This will be initiated as soon as the screen in Task4 is completed.

Task 6. The identified breast targeted transduction peptides (maximum of 2) will be examined for their ability to deliver marker complexes into breast cancers in nude mouse xenograft models following intra-tumoral (local) as well as intra-peritoneal (systemic) administration. Nude mice will be inoculated subcutaneously with MCF-7 cells. When the tumors reach palpable size, the peptide-avidin-ß-gal complexes will be injected intra-tumorally as well as intravenously and the extent of transduction examined three hours post-injection for the intra-tumoral injection and 6 hours post-injection for the intravenous injection by X-gal staining of tumor sections and by quantitating ß-gal activity in tumor lysates. The TAT PTD and PTD-5 peptides will be used as a positive control. 3 mice will be used per treatment group. However, different doses of peptide-marker conjugates may have to be tested. (Months 6-12, Year 2).

Progress: This will be initiated as soon as Task 5 is completed.

Objective 2: To examine the ability of peptide mediated transduction of specific agents to regulate breast cancer cell growth and apoptosis.

Task 1. The ability of the 2 optimal cationic and the 2 optimal breast targeted transduction peptides to deliver three different potentially therapeutic peptides will be tested. The three peptides to be tested include one derived from the amino terminus of Smac, a pro-apoptotic protein, a c-terminal p53 peptide able to activate the transcriptional activity of wildtype p53 as well as certain p53 mutants, a peptide able to block NF-kB activation (NBD), and a general pro-apoptotic factor able to disrupt mitochondria. PTD-5 fused to the Smac, p53, NBD and KLAK peptides will be used as positive controls. The peptide fusions will be examined for ability to inhibit viability or proliferation of breast cancer cells in culture. Increasing concentrations of the different peptide fusions will be added to the media and cell viability measured at different time points by MTT and by FACS analysis following PI and annexin V staining. To examine the tissue specificity of the observed effects, the activity of the peptides will be compared in HeLa, LNCaP and Saos-2 cells. (Months 1-12, Year 2)

Progress: We choose to use peptides based on the amino terminus of mature Smac, a protein demonstrated to enhance apoptosis by blocking IAP family members. The first set of peptides consisted of the protein transduction domain PTD5 linked to either the first 34 amino acids of mature Smac via a diglycine bridge. PTD5-Smac34 both enhanced TRAIL- and etoposide-mediated death, but unlike the results in prostate tumors, was unable to induce cell death on its own. Cell death resulted from apoptosis as shown by Annexin-V staining and Western analysis of PARP cleavage. In an effort to optimize the Smac peptides, a panel of protein transduction domains, (PTD), was tested for their ability to transduce MCF-7 and ZR75-1 cells. 8K proved to be the most efficient PTD for this task. Thus, we synthesized new peptides containing either the first 12 or 34 amino acids of mature Smac linked to 8K via a diglycine bridge, (Smac12-8K, Smac34-8K respectively). Smac34-8K efficiently induced and enhanced apoptosis mediated by etoposide and Trail, whereas Smac12-8K remained ineffective. Taken together, these experiments establish that peptides based on the first 34 amino acids of mature Smac may be a better therapeutic tool than shorter Smac peptides that only encompass the first 7-12 amino acids. Given that we know have shown that 12K and 12R peptides are more effective, we are now synthesizing 12K-Smac24 and 12R-Smac34.

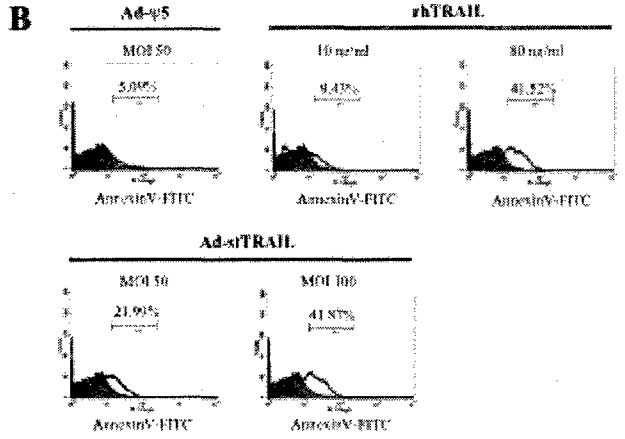
Task 2. To ability of the p53-terminal peptides to induce endogenous p53 transcriptional activity will be examined by transfection of the breast cancer and non-breast cancer cells with a p53-dependent luciferase reporter followed by addition of increasing concentrations of the p53 peptide fusions. The level of luciferase will be measured 6 hours post-addition of the peptide. (Months 1-6, Year 2)

Progress: Work from our lab as well as work performed in the laboratories of several collaborators suggest that the effects of the c-terminal p53 peptide may not be sequence dependent, but instead may be based on a charge effect. Thus we are not pursing the p53 c-terminal peptide as a therapeutic agent.

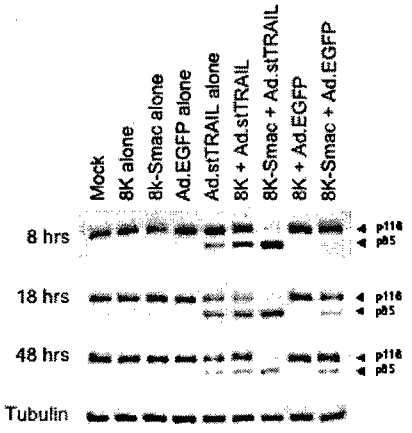
Task 3. The ability of the p53, NBD and Smac peptides to sensitize the tumor lines to the apoptotic effects of rTRAIL, etoposide and radiation will be examined. Increasing concentrations of the various peptide

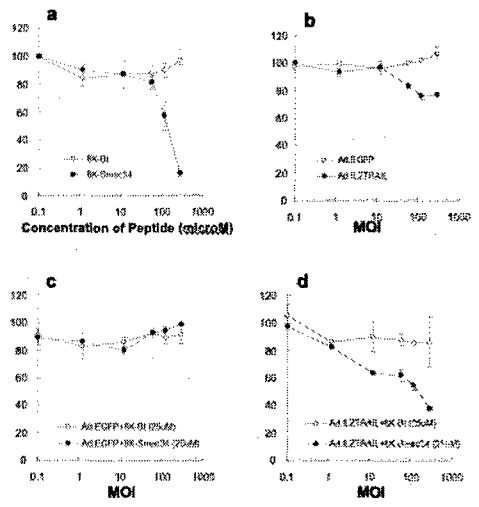
fusions will be added to cells followed by addition of suboptimal doses of rTRAIL and etoposide, as well as radiation. (Months 6-12, Year 2; Months 1-6, Year 3)

Progress: In addition to performing studies with recombinant TRAIL protein, we have constructed adenoviral vectors expressing human membrane bound TRAIL as well as a soluble TRAIL. Transduction of a variety of tumor cells including MCF-7 breast tumor cells resulted in an increase in apoptosis in both the soluble and membrane bound groups as determined by Annexin staining.



Interestingly, in preliminary in vivo experiments, the membrane bound TRAIL was far more effective than the soluble TRAIL in reducing tumor size. We also have shown that gene transfer of Ad.TRAIL was able to synergize with the 8K-Smac34 peptide to induce apoptosis in breast tumor cells in culture. Since it has been shown that the PTDs can function to enhance adenoviral infection if the virus and PTD are co-mixed, we have used the 8K-Smac34 PTD to enhance adenoviral infection, resulting in a significantly better transduction and anti-tumor effect. In this experiment, cells were shown to be sensitive to high dose of the PTD-Smac (panel A) or to a high MOI of the soluble TRAIL virus (Panel B). When a suboptimal dose of the PTD-SMAC or control PTD was used with the sTRAIL virus (Panel D), substantially higher apoptosis was observed at a low MOI compared to that observed with the Ad.eGFP control adenovirus (Panel C). We also have confirmed this effect by measuring the extent of PARP cleavage following addition of Ad.sTRAIL, Smac34-8K or both mixed together. Thus, we are now using PTDs both to improve gene transfer and to sensitize the cells to TRAIL-mediate apoptosis.





Key Research Accomplishments of the first year:

1. Optimized protein transduction of breast tumor cells, identifying the optimal PTD.
2. Demonstrated that PTD-Sma34 worked in conjunction with Ad.TRAIL gene transfer to induce breast tumor apoptosis.
3. Demonstrated that membrane bound TRAIL worked more effectively than soluble (secreted) TRAIL.

4. Demonstrated that co-administration of Ad.TRAIL with PTD-Smac34 resulted in not only enhanced adenoviral transduction of the tumors, but resulted in a stronger apoptotic effect.

Reportable Outcomes.

The results of the PTD optimization, TRAIL sensitivity of breast tumors and enhancement of adenoviral transduction are all being written up for publication. Drafts of these manuscripts will be provided as soon as they are accepted for publication.